In situ hybridization with fluoresceinated DNA

Joop Wiegant, Thomas Ried, Petra M.Nederlof, Mels van der Ploeg, Hans J.Tanke and Anton K.Raap
Department of Cytochemistry and Cytometry, 72 Wassenaarseweg, 2333 AL Leiden, The Netherlands

Received April 9, 1991; Revised and Accepted May 8, 1991

ABSTRACT

We have used fluorescein-11-dUTP in a nick-translation format to produce fluoresceinated human nucleic acid probes. After *in situ* hybridization of fluoresceinated DNAs to human metaphase chromosomes, the detection sensitivity was found to be 50 – 100 kb. The feasibility and the increase in detection sensitivity of microscopic imaging of *in situ* hybridized, fluoresceinated DNA with an integrating solid state camera for rapid cosmid mapping is illustrated. Combination of fluoresceinated DNA with biotinated and digoxigeninated DNAs allowed easy performance of triple fluorescence *in situ* hybridization. The potential of these techniques for DNA mapping, cytogenetics and biological dosimetry is briefly discussed.

INTRODUCTION

During the last ten years, a number of non-radioactive nucleic acid probe modification procedures has been developed. *In situ* hybridization with such modified probes provides high spatial resolution as well as high detection sensitivity (1–6). Furthermore, it is possible to visualize different targets simultaneously (7–9).

In the majority of the methods described for conventional DNA or RNA recombinant probes, an element (i.e. a hapten) is introduced in the nucleic acid probes, either (photo)chemically or enzymatically, that renders them detectable by affinity cytochemistry (for reviews, see 10, 11). Only few reports describe the direct coupling of reporter molecules like fluorochromes or enzymes to nucleic acid probes.

Application of 3'-end fluorochromized RNA probes, using periodate oxidized RNA and the thiosemicarbazide derivatives of fluorescein or rhodamine, was described in 1980 by Bauman et al (12). The rather low sensitivity of the method, attributed to the presence of only one fluorochrome molecule per hybridization probe fragment, was increased by employing anti-fluorescein antibodies for subsequent immunocytochemical detection (13, 14). In 1986, the use of a fluorescein sulfhydryl ligand to visualise target sequences that had been hybridized *in situ* with mercurated DNA probes was reported by Hopman et al (15). The feasibility of this procedure, which formally is not a true direct one, but does not employimmunocytochemical detection principles, was demonstrated in the localization of mouse satellite DNA sequences in metaphase chromosomes and interphase nuclei from human-mouse hybrid cell lines (16). The limits of sensitivity of this 'direct' *in situ* hybridization procedure, have not been explored.

An advantage of fluorochrome- or enzyme-labelled probes is that no immunocytochemical visualization procedure is necessary, resulting in low backgrounds. General drawbacks of the direct methods described, however, are that they probably are less sensitive than indirect methods applying biotin-, digoxigenin- or other hapten-modified probes and that they do not fit nucleic acid labelling formats that are widely used in molecular biology.

In this paper we present experiments which show the potential of a newly developed reagent, fluorescein-11-dUTP, for *in situ* hybridization experiments.

MATERIALS AND METHODS

Cells and chromosomes

Metaphase chromosome spreads were produced from phytohemagglutinin stimulated normal and 4 Gy irradiated human peripheral blood lymphocyte cultures according to routine procedures. Nuclei from the T24 bladder cancer cell line (17) were obtained similarly.

DNAs

The following human genomic recombinant DNAs have been used: the satellite III probe pUC-1.77 for 1q12 (18), the alphoid probes p308 (19) and p17H8 (20) for the centromeres of chromosomes 6 and 17, respectively; Bluescript libraries for chromosomes 2, 4 and 8; 9 phage clones spanning 105 kb of target in the c-*myc* region on 8q24 (21); and a 30 kb cosmid probe from the human *mdr*-1 gene on 7q21.

Nick-translation and probe storage

Probes were labelled with either biotin-11-dUTP (Sigma) or digoxigenin-11-dUTP (Boehringer-Mannheim) or fluorescein-11-dUTP (a generous gift from Boehringer-Mannheim) by nick-translation according to routine procedures (22).

The satellite probes were ethanol precipitated and dissolved in 60% deionized formamide, 2×SSC (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 50 mM phosphate, pH 7.0, containing per μl 10 ng probe, 500 ng sonicated salmon spermDNA and 500 ng yeast tRNA. The cosmid probe, the phage probes and the chromosome libraries were stored at −20° C in

10 mM Tris-HCl, 1 mM EDTA, pH 7.8 (TE) with a 50-fold excess of sonicated salmon sperm DNA and yeast tRNA.

In situ hybridization

Pretreatment. Before *in situ* hybridization the slides were pretreated with RNase and pepsin as described (23).

Repetitive DNAs. The satellite DNAs were used at 2 ng/μl. Five μl of probe solution was brought on each slide and covered with a 18×18 mm^2 coverslip. Then the slides were put on a 80°C hot plate for 3 min, after which the hybridization was allowed to proceed overnight at 37°C. The slides were washed 3×5 min with 60% formamide, 2×SSC at 37°C, followed by 2×5 min washes with 2×SSC.

Phage- and cosmid DNA hybridizations. Phage- and cosmid probes were hybridized according to the competition principle (5, 24). Briefly, 2 ng/μl labelled DNA in 50% formamide, 2×SSC, 50 mM phosphate, 10% dextran sulfate, pH 7 was pre-annealed for 2 hrs at 37°C to 1000 ng/μl fragmented total human placental DNA. Ten μl was brought on a denatured slide (see below) under a 18×18 mm^2 coverslip, which was subsequently sealed with rubber cement. After overnight hybridization at 37°C and removal of the rubber cement, the slides were immersed in 50% formamide, 2×SSC, pH 7 at 45°C to loosen the coverslips. Then the slides were washed 3×5 min with 50% formamide, 2×SSC, pH 7 at 45°C, followed by 3×5 min with 0.1×SSC at 60°C. In case of immunocytochemical amplification, slides were washed with the appropriate immunocytochemical buffer.

Denaturation of *in situ* DNA was achieved by placing on the slide, 100 μl of 70% deionized formamide, 2×SSC, 10 mM phosphate pH 7.0 under a 24×50 mm^2 coverslip, after which it was placed on a 80°C hot plate for 3 min. The coverslip was removed and the slide immersed in 70% ethanol at −20°C for 5 min. Next, the slide was dehydrated at RT (1×5 min 90% and 1×5 min 100% ethanol) and air-dried at 37°C.

Chromosome DNA libraries. For the simultaneous hybridization of fluoresceinated chromosome 8, biotinated chromosome 4 and digoxigeninated chromosome 2 DNA libraries, the appropriate amounts of DNAs in TE-buffer were mixed and precipitated with ethanol in the presence of a 500-fold excess of fragmented total human placental DNA. The DNAs were dissolved in 50% deionized formamide, 2×SSC, 50 mM phosphate, pH 7.0, 10% dextran sulfate and denatured for 5 min at 75°C. After chilling on ice, the repetitive sequences were competed out for 2 hrs at 37°C. Next, the pre-annealed libraries were mixed, *in situ* hybridized and washed as described for phage- and cosmid DNAs.

Immunocytochemical procedures

Immunocytochemical amplification of fluorescein-DNA. The slides were briefly washed with 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% (w/v) Tween-20 (TNT) and blocked for 20 min at 37°C with 0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.5% (w/v) Boehringer blocking reagent (TNB), incubated for 45 min at 37°C with rabbit anti-fluorescein antibody (Dakopatts), followed by a second incubation with FITC-conjugated goat anti-rabbit antibody (Sigma). The dilutions of the antibodies were made in TNB. Washes were 3×5 min with TNT.

Triple hybridization, one-step procedure. The slides were briefly washed with TNT and blocked with TNB for 20 min at 37°C. After a brief wash with TNT, they were incubated for 45 min at 37°C with a mixture of avidin-D-AMCA (Vector) and sheep-anti-digoxigenin-TRITC (Fab fragments, Boehringer), diluted in the blocking medium. Then, the slides were washed 3×5 min with TNT.

Triple hybridization, four-step procedure. The slides were blocked with 4×SSC, 5% non-fat dry milk (4M), then incubated with avidin-D-AMCA in 4M for 20 min at RT. After 2×5 min washes with 4×SSC, 0.05% (w/v) Tween-20 and a 1×5 min wash with TNT, a second incubation with a mixture of rabbit-anti-FITC, biotinated goat-anti-avidin-D and mouse-anti-digoxigenin (Boehringer), diluted in TNB for 30 min at 37°C, was performed. The third incubation was with a mixture of avidin-D-AMCA, sheep-anti-mouse-Dig andgoat-anti-rabbit-FITC, diluted in TNB for 30 min at 37°C. Finally, the slides were incubated with sheep-anti-digoxigenin-TRITC, diluted in TNB for 30 min at 37°C. Washes were 3×5 min with TNT.

Microscopy

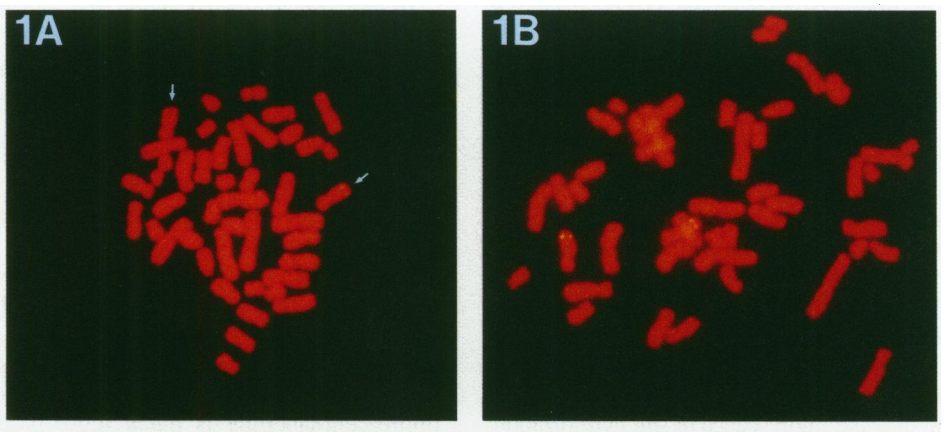
The slides were dehydrated through an ethanol series, air-dried and embedded in medium consisting of 9 parts glycerol and 1 part 1 M Tris-HCl, pH 7.5, containing 2% 1,4-diazabicyclo-[2,2,2]-octane as an anti-fading reagent. In case of a single hybridization, it also contained propidium iodide (1 μg/ml) as a general DNA counterstain. A Leitz Dialux Epifluorescence microscope equipped with a 100 W mercury arc lamp and appropriate filter sets for red, green and blue fluorescence (8) was used. A 63×lens with a numerical aperture of 1.3 was generally used for photography to 640 ASA 3M color slide films.

Digitalization of the *in situ* hybridization images was carried out by a cooled charged coupled device (CCD) camera (Photometrics) employing a Kodak chip of 1348×1037 elements, which was mounted on the fluorescence microscope described above. Image recording and processing was performed using the TCL software package (Multihouse) running on a SUN Workstation interfaced to the camera.

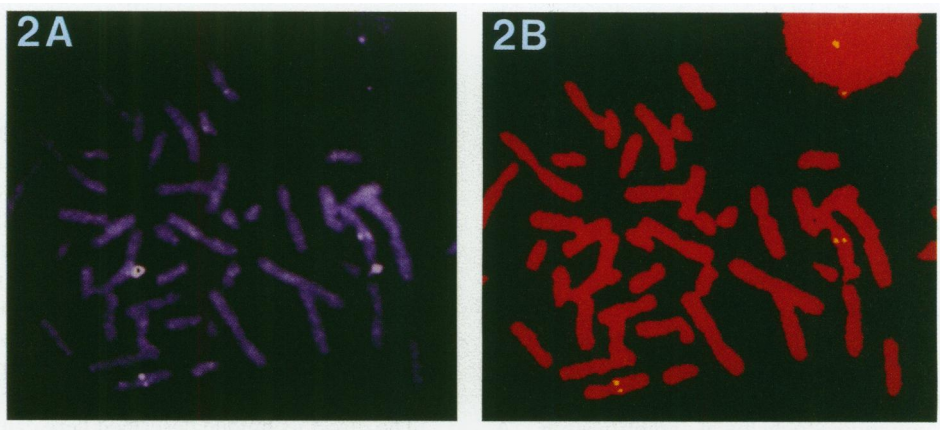
RESULTS

In order to estimate the detection sensitivity of fluoresceinated DNA probes in fluorescence *in situ* hybridization we used in preliminary experiments a series of human DNAs which recognize targets varying in copy number from high (i.e. a number of alpha satellite DNAs) to moderate (i.e. rDNA). All these targets could be visualized readily. To determine the detection sensitivity more accurately we used a set of phage clones spanning the c-*myc* region of chromosome 8. Only when the size of the target DNA was lower than about 50 kb, signals were no longer visible by conventional fluorescence microscopy. Figure 1A shows the *in situ* hybridization result obtained with 105 kb of fluoresceinated phage DNA for the c-*myc* region on 8q24 without immunocytochemical amplification. Each of the sister chromatids of the two homologue chromosomes diplays the fluorescein signal. Figure 1B shows the hybridization with the same probe after immunocytochemical amplification and illustrates the increase in intensity obtainable with anti-fluorescein antibodies. Also, the direct detection appears to provide somewhat better resolution.

In these and similar experiments with probes for 1−5 kb targets, it was consistently observed that in the indirect mode, background is introduced at the immunocytochemical level. This



Figures 1A-1B. Fluorescence *in situ* hybridization with fluoresceinated-phage DNA spanning 105 kb of target sequence around the c-myc region on chromosome 8 to human metaphase chromosomes, without (1A) and with (1B) immunocytochemical amplification of signal. A few background spots, resulting from the immunocytochemical amplification reaction, may be noted on the chromosomes.



Figures 2A-2B. Digital image acquisition and image processing of fluorescence *in situ* hybridization results. Fluoresceinated cosmid DNA spanning 30 kb of genomic target at 7q21 was hybridized *in situ* to normal human metaphase chromosomes. **2A**. The primary green image obtained after 10 sec of integration, photographed from the monitor screen. **2B**. The image obtained after local background subtraction, removal of non-chromosomal background spots, contrast stretching and reduction of the number of grey levels to three by thresholding the grey value image at two different levels to obtain the chromosomes (artificially colored red) and the hybridization spots (artificially colored yellow). Two autofluorescent dirt particles were present in this microscopic field.

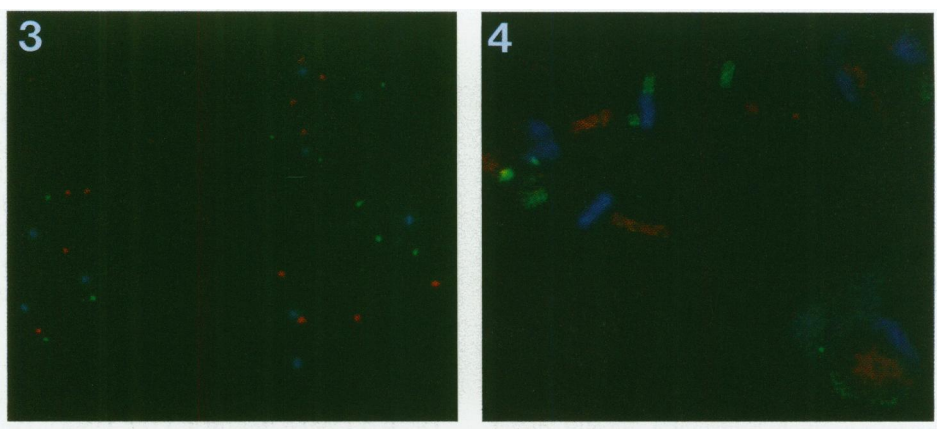


Figure 3. Triple fluorescence *in situ* hybridization to repetitive DNA targets in three interphase nuclei from a bladder cancer cell line. In each nucleus, the three green signals derive from the fluorescein-labelled probe for chromosome 6 (p308), the three blue AMCA signals from the biotin-labelled probe for chromosome 1 (pUC1.77) and the four red TRITC signals from the digoxigenin-labelled probe for chromosome 17 (p17H8). No counterstaining, triple exposure photomicrograph. Due to low autofluorescence levels, the contours of the nuclei are hardly visible on this photomicrograph.

Figure 4. Immunocytochemically amplified triple fluorescence *in situ* hybridization with chromosome specific DNA libraries to irradiated human lymphocyte meta- and interphase cells. Green signals derive from the fluoresceinated chromosome 8 library, blue AMCA signals from the biotin labelled chromosome 4 library and red TRITC signals from the chromosome 2 library. No counter-staining, triple exposure photomicrograph. Note the spatial distribution of the chromosomes in the three interphase nuclei.

fact was substantiated by immunocytochemical TRITC detection of the fluoresceinated DNAs.

To employ the low noise of the direct detection we have used digital imaging with an integrating camera (25, 26). Thephotographs of Figures 2A and 2B represent the results obtained with a fluoresceinated cosmid DNA located at 7q21 and a 10 sec integration time. These signals were not visible by eye only. Similar experiments using indirect detection principles with either biotin, or digoxigenin, or fluorescein showed quite some background spots on top of the weakly autofluorescing chromosomes, as well as on the glass. Experiments with smaller probes suggest that the detection sensitivity of fluoresceinated DNA probes can be increased by at least a factor of 30 using this type of instrumentation.

To show the potential of triple fluorescence *in situ* hybridization with fluoresceinated, biotinated- and digoxiginated probes and its application in cancer cytogenetics and biological dosimetry, we performed such hybridizations to cancer cells and irradiated lymphocytes.

Figure 3 shows the results of a triple *in situ* hybridization with three satellite DNA probes for chromosomes 1 (blue), 6 (green) and 17 (red) to interphase nuclei of T24 bladder cancer cells. The fluorescein-DNA was not amplified immunocytochemically, while the biotin- and digoxigenin labelled probes needed only one immunocytochemical incubation for visual detection to AMC-A and TRITC, respectively. The numerical aberrations seen in the interphase cells are obvious and in accordance with the karyotype.

Figure 4 shows the result obtained with triple *in situ* hybridization with three chromosome DNA libraries to 400 rad irradiated human lymphocytes using the four-step immunocytochemical procedure for visualization of all three haptens. However, fluoresceinated library DNAs are visible in the microscope without immunocytochemical amplification. A number of translocations can easily be detected in the metaphase shown. Also note the hybridization domains in the interphase nuclei. Since the standard, biomedical fluorescence microscope we have used, allows selection of only three colors, total DNA counterstaining could not be performed in the experiments of Figures 3 and 4, without obscuring the specific hybridization signals.

DISCUSSION

The results clearly show the favorable features of the use of fluorescein-11-dUTP for *in situ* hybridization experiments. Firstly, the labelling with fluorescein-dUTP can be performed routinely with DNA polymerase reactions; secondly, it yields very low background after *in situ* hybridization; thirdly, the direct detection of *in situ* hybrids is of good sensitivity and when necessary, this can readily be increased by employing the fluorescein moiety as a hapten in an amplification reaction or integrating camera systems; fourthly, fluoresceinated probes can be combined conveniently with biotinated- and digoxiginated probes for triple fluorescence *in situ* hybridization. Finally, direct detection of fluoresceinated DNA appears to give better resolution than indirect detection.

In a separate paper we report on the use of fluorescein-dUTP in terminal deoxynucleotide transferase reactions for fluorochromizing synthetic oligonucleotide and their application in multiple *in situ* mRNA detection (27). Studies with red, respectively blue fluorescing rhodamine- and coumarin derivatives of dUTP are being undertaken.

In the direct, visual mode the sensitivity of detection of fluoresceinated DNA probes was 50−100 kb in our experience (Figure 1A). In the indirect, visual mode it equals the one of immunocytochemical detection of biotinated or digoxigenated probes and is in the range of 1−5 kb. The lowest level of detection using integrating imaging is currently under study, but as shown by the result of Figures 2A−2B is more than sufficient for application in mapping DNAs contained in e.g. cosmid vectors.

Former publications from our Department on multiple fluorescence *in situ* hybridizations (7−9) reported the use of various combinations of the biotin, acetylaminofluorene, mercury/hapten and sulfonation/transamination labelling procedures. The combination of biotin-, digoxigenin- and fluorescein-labelling is much more practical, because of the identical labelling formats and hybridization conditions. Furthermore, by simply mixing the haptenized dUTPs in the nick-translation it proved possible to label one target with two or three fluorochromes. Also by mixing single-labelled probes for the same target, multi-color labelling of one target is possible. With these approaches, the multiplicity of *in situ* hybridization can be increased considerably (9).

There are many applications of the *in situ* hybridization techniques described. A currently important one is found in DNA mapping. Given the high molecular resolution and the fact that genomic probes need not to be free of repetitive elements for good signal to noise chromosomal localization (5, 24) rapid, simultaneous multicolor localization of several phage-, cosmid- or yeast artificial chromosome clones is feasible. In tumor cytogenetics, indirect *in situ* hybridization techniques now are applied regularly for the rapid assessment of numerical chromosome(segment) aberrations in interphase cells ('interphase cytogenetics') using satellite DNA and cosmid probes (28).

The direct technique described here can be readily applied for such purposes, and when combined with biotin or digoxigenintechniques, sophisticated interphase cell analysis is possible. In this respect, the increase in speed with which *in situ* hybridization experiments using fluoresceinated probes can be performed in the direct mode, is a significant advantage over the indirect techniques using haptens like e.g. biotin or digoxigenin.

Recently, we and others have shown that by using numerical and color information of *in situ* hybridization, also structural aberrations like e.g. the Philadelphia chromosome, can be identified in interphase cells (29, 30). Also, multi-color *in situ* hybridization with chromosome library DNAs will prove to be of value in analyzing complex karyotypic changes, which are difficult to asses by banding techniques. Finally, the technique is of importance for biological dosimetry as it allows sensitive and easy scoring of chromosomal aberrations.

ACKNOWLEDGEMENTS

The authors wish to thank Dr M.Lipp for the phage clones; Dr H.J.Cooke for pUC1.77 DNA; Dr H.F.Willard for the alphoid DNAs; Dr J.W.Gray for the Bluescript chromosome libraries; Dr F.Baas for the MDR cosmid probe; Dr F.Darroudi for the irradiated lymphocytes, and Boehringer-Mannheim GmbH for the sample of fluorescein-11-dUTP. This research was supported in part by the Foundation for Medical Health Research (Medigon), grant no. 900-534-060 and The Netherlands Organization for Scientific Research (NWO) grant no. PGS 90.129.

REFERENCES

1. Landegent,J.E., Jansen in de Wal,N., Van Ommen,G.J.B., Baas,F., De Vijlder,J.J.M., Van Duijn,P. and Van der Ploeg,M. (1985) *Nature* **317**, 175–177.
2. Lawrence,J.B., Villnave,C.A. and Singer,R.H. (1988) *Cell* **52**, 51–61.
3. Lawrence,J.B., Singer,R.H. and McNeil,J.A. (1990) *Science* **249**, 928–931.
4. Trask,B., Pinkel,J.D. and Van den Engh,G. (1989) *Genomics* **5**, 710–717.
5. Lichter,P., Tang,C.C., Call,K., Hermanson,G., Evans,G., Housman,D. and Ward,D.C. (1990) *Science* **247**, 64–69.
6. Bhatt,B., Burns,J., Flannery,D. and McGee,J.O.D. (1988) *Nucl. Acids Res.* **16**, 3951–3961
7. Hopman,A.H.N., Wiegant,J., Raap,A.K., Landegent,J.E., Van der Ploeg,M. and Van Duijn,P. *(1986) Histochemistry* **85**, 1–4.
8. Nederlof,P.M., Robinson,D., Abuknesha,R., Wiegant,J., Hopman,A.H.N., Tanke,H.J. and Raap,A.K. (1989) *Cytometry*10, 20–27.
9. Nederlof,P.M., Van der Flier,S., Wiegant,J., Raap,A.K., Tanke,H.J., Ploem,J.S. and Van der Ploeg,M. (1990) *Cytometry* **11**, 126–131.
10. Raap,A.K., Hopman,A.H.N. and Van der Ploeg,M. (1989) In Bullock,G. and Petrusz,P. (eds.), Techniques in Immunocytochemistry Vol IV, Academic Press pp 167–198.
11. Raap,A.K., Dirks,R.W., Jiwa,N.M., Nederlof,P.M. and Van der Ploeg,M. (1990) In Racz,P., Haase,A.T. and Gluckman,J.C. (eds.), Modern Pathology of AIDS and other retroviral infections. Eds: Karger, Basel, pp 17–28.
12. Bauman,J.G.J., Wiegant,J., Borst,P. and Van Duijn,P. (1980) *Exp. Cell Res.* **138**, 485–490.
13. Schmitz,H. and Kampa,D. (1979) *J. Immunol. Meth.* **26**, 173–174.
14. Bauman,J.G.J., Wiegant,J. and Van Duijn,P. (1981) *Histochemistry* **73**, 181–193.
15. Hopman,A.H.N., Wiegant,J., Tesser,G.I. and Van Duijn,P. (1986) *Nucl. Acids Res.* **14**, 6471–6488.
16. Hopman,A.H.N., Wiegant,J. and Van Duijn,P. (1987) *Exp. Cell Res.* **169**, 357–368.
17. Bubenik,J., Raresova,M., Vicklichy,V., Jakoubkova,J., Sainerovac,C. and Donner,J. (1973) *Int. J. Cancer* **11**, 765–771.
18. Cooke,H.J., Hindley,J. *(1979) Nucl. Acids Res.* **6**, 3177–3197.
19. Jabs,E.W., Wolf,S.F. and Migeon,B.R. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 4884–4888.
20. Waye,J.S. and Willard,H.F. (1986) *Nucl. Acids Res.***14**, 6915–6927.
21. Henglein,B., Synovzik,H., Groltl,P., Bomkamm,G.W., Hartl P. and Lipp,M. (1989) *Mol. Cell. Biol.* **9**, 2105–2113.
22. Wachtler,F., Hartung,M., Devictor,M., Wiegant,J., Stahl,A. and Schwarzacher,H.G. (1989) *Exp. Cell Res.* **184**, 61–71.
23. Wiegant,J., Galjart,N., Raap,A.K. and d'Azzo,A. (1991) *Genomics*, in press
24. Landegent,J.E., Jansen in de Wal,N., Dirks,R.W., Baas,F., and Van der Ploeg,M. (1987) *Hum. Genet.* **77**, 366–370.
25. Aikens,R.S., Agard,D.A. and Sedat,S.W. (1989) *Meth. Cell Biol.* **29**, 291–315
26. Viegas-Pequignot,E., Dutrillaux,B., Magdelenat,H. and Coppey-Moisan,M. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 852–586
27. Dirks,R.W., Van Gijlswijk,R.P.M., Vooijs,M.A., Smit,A.B., Bogerd,J., Van Minnen,J., Raap,A.K. and Van der Ploeg,M. (1991) *Exp. Cell Res.* in press
28. Arnoldus,E.P.J., Noordermeer,I.A., Peters,A.C.B., Voormolen,J.H.C., Bots,G.T.A.M., Raap,A.K. and Van der Ploeg,M. (1991) *Genes Chromosomes and Cancer* **3**, 101–107.
29. Arnoldus,E.P.J., Wiegant,J., Noordermeer,I.A., Wessels, J.W., Beverstock,G.C., Grosveld,G.C., Van der Ploeg,M. and Raap,A.K. (1990) *Cytogenet. Cell Genet.* **54**, 108–111
30. Tkachuk,D.C., Westbrook,C.A., Andreeff,M., Donlon,T.A., Cleary,M.L., Suryanarayan,K., Homge,M., Redner,A., Gray,J. and Pinkel,D. (1990) *Science* **250**, 559–562